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EFFECT OF CAFFEINE ON SELECTED REPRESENTATIVES OF PHOTOTROPHIC MICROORGANISMS

Lenka Hutárová, Miroslava Hlebová, Simona Bérešová, Dominika Vešelenyiová*

Address(es): RNDr. Lenka Hutárová, PhD.,
University od Ss. Cyril and Methodius in Trnava, Institute of Biology and Biotechnology, Department of Biology, Nam. J. Herdu 577/2, 971 01 Trnava, Slovakia, +421335565 337.

*Corresponding author: lenka.hutarova@ucm.sk

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ABSTRACT

Aquatic ecosystems are becoming increasingly affected by micropollutants. The survival of microbial communities and entire aquatic ecosystems depends directly on their ability to adapt to this type of pollution. One of the most common micropollutants found in water bodies, especially in the vicinity of large cities, is caffeine. This stimulant directly affects living organisms and can cause changes in the species composition of microbial communities. However, the best way to decontaminate caffeine from the environment may be through the use of microorganisms. Therefore, in this study, the effect of caffeine on selected species of cyanobacteria and algae was investigated. The following strains of cyanobacteria: *Geitlerinema cf. acuminatum* (CCALA 141), *Chlorogloeopsis fritschii* (CCALA 039), *Chlorogloeopsis fritschii* (CCALA 1005), and *Synechococcus granulates* (CCALA 187) and a eukaryotic photosynthetic flagellate *Euglena gracilis* (strain Z) were selected. Probit analysis determined the minimum inhibitory concentration (MIC) of caffeine for each studied species. Also, the inhibitory effect of caffeine on each tested strain was monitored *in vivo*. Next, the strains were microscopically observed, and the potential change in their morphology under the caffeine treatment was recorded. The reproduction rate of all species in the study were moderately inhibited by caffeine, but during the cultivation, they were able to grow in comparison with the control sets. The most sensitive species was *Geitlerinema cf. acuminatum* (CCALA 141). The study also showed a significant effect of caffeine on morphology changes in the strains under investigation. Caffeine at low concentrations also showed a stimulating effect on the growth of the studied species. This may lead to their improved competence potential within microbial communities in the aquatic biotopes.

Keywords: caffeine, *Geitlerinema* spp., *Chlorogloeopsis* spp., *Synechococcus* spp., *Euglena gracilis*, growth inhibition

INTRODUCTION

Caffeine is one of the most widely used stimulants in the world. For example, caffeine is one of the most consumed stimulants in the United States (**Nawrot *et al*., 2003**), where men ingested about 243 mg/day and women 251 mg/day (**Knapik *et al.*, 2022**). In addition, it is one of the most common compounds in pharmaceutical and personal care products (PPCP) (**Ondarza *et al*., 2019**). Its high use predicted its presence in the environment, including aquatic biotopes. It represents the major water pollutant in all types of water bodies with concentrations around 0.007 to 49.6 µg/L in river systems, 0.02 to 37.48 µg/L in lakes, 0.02 to 1.54 µg/L in pools and 0.002 to 0.10 µg/L in the sea (**Li *et al*., 2020a**). Caffeine is monitored in the environment despite its low levels and the efficient removal of >99% of the caffeine from wastewater (**Strauch *et al*., 2008**). Its presence can be used as an indicator of anthropogenic activity (**Dafouz *et al*., 2018**) and an indicator of wastewater input to surface waters (**Bruton *et al*., 2010**). The occurrence of this contaminant in aquatic biotopes has a significant impact on aquatic ecosystems. Substantial reductions in microbial biomass (up to 25%) and significant changes in microbial composition can occur when communities are exposed to this particular contaminant (**Zhang *et al*., 2016**). The number of cyanobacterial species in microbial biofilms decreased at caffeine concentrations around 10 µg/L. Concentrations of 5 µg/L caused algal biomass to decrease but did not affect cyanobacteria (**Lawrence *et al.*, 2012**). Also, in studies on natural periphyton and biofilms, exposure to caffeine (up to 300 µg/L) caused an increase in cyanobacterial species (**Dunck *et al*., 2015; De Sousa *et al*., 2021**). Caffeine has an inhibitory effect on the growth of cyanobacteria and algae at specific concentrations. Caffeine synergises with many DNA-damaging agents (**Lau and Pardee, 1982**) in various organisms due to specific inhibition of ATM/ATR kinases (**Zhou *et al*., 2000**). The morphology of some phototrophs can also be targeted. In heterocystous cyanobacteria such as *Anabaena doliolum*, higher concentrations of caffeine (around 200 µg/L) cause the akinete formation to start later but do not affect heterocyte formation. Akinets then show higher UV resistance (**Srivastava *et al*., 1971**). In the case of *Euglena gracilis*, the presence of caffeine at a concentration of 10 nM could have an inhibitory effect on the repair of nucleus DNA damage caused by UV radiation (**Nicolas *et al*., 1980**). Studies on different algal species, such as *Chlorella homosphaera* and *Scenedesmus obliquus*, show that caffeine in low doses does not affect the growth of these species and does not pose the high risk to water phototrophs in incubation found in nature. Caffeine may also reduce the toxicity of other water pollutants, such as ciprofloxacin when applied to algal cultures (**Diniz *et al*., 2021**).

This study aimed to evaluate the effect of caffeine on the growth and possible morphological changes of several species of cyanobacteria and a strain of algae. First, the minimum inhibitory concentration of caffeine was determined. The percentage growth of cyanobacteria and algal strains treated with caffeine was studied, and finally, the potential effect of caffeine on cyanobacterial and algal morphology was evaluated.

MATERIAL AND METHODS

Phototrophic microorganisms used and growth condition

This study used selected species of cyanobacteria: *Geitlerinema cf. acuminatum* (CCALA 141), *Chlorogloeopsis fritschii* (CCALA 039), *Chlorogloeopsis fritschii* (CCALA 1005), and *Synechococcus bigranulatus* (CCALA 187) obtained from Culture Collections of Autotrophic Organisms (CCALA) (Třeboň, Czech Republic). From the algae, *Euglena gracilis* (strain Z) was selected. This strain belongs to the collection of microorganisms at the Department of Biology, UCM in Trnava. For the genus *Euglena*, Cramer-Myers liquid culture medium (CM) **(Cramer and Myers, 1952)** was used. Cyanobacterial strains were cultivated in BG 11 medium **(Stanier *et al.*, 1971)** with pH 7.5. Triplicate cultures of each strain were started by adding 10 mL of sterile BG11 and CM medium (for *Euglena gracilis*) to 1 mL of cyanobacteria and algae inoculum to sterile tubes. Then they were incubated for 5 days at 23 °C and with 24h illumination (light intensity 48,6 µmol. foton m^{-2} s^{-1}).

Inoculum preparation

Before the inoculum preparation, the studied strains were cultivated as is described above. The inoculum for each strain was prepared by adding 1 mL of medium BG11 and CM containing the strain to 4 mL of fresh medium BG11 and CM medium to a final concentration 2x10^6 cell/mL
10^6 cells/mL according to **Čížková *et al*. (2019)** and then the density was adjusted to 0.8 McFarland by densitometer (Densi-la-meter II, Erba Lachema s.r.o. Brno).

***In vitro* inhibitory effect of caffeine analysis and minimum inhibitory concentration (MIC) determination**

The sterile 96-well microtiter plates were used for growth inhibitory effect of caffeine (MP Biomedicals) determination according to **Hutárová *et al*. (2023)** with small modification. Each well of 96-well microtiter plates contained 100 µL of BG11 medium or CM (for *Euglena gracilis*) (lines from A to E and line H). 200 µL was the final volume of each well. The line F represent the purity control of medium (200 µL) and line G served as growth control of tested phototrophs (100 µL of growth medium + 100 µL of inoculum). Caffeine was diluted at an initial concentration of 100 mg/mL in the growth medium and 100 µL of this suspension was added to the first column (in line A-E). Next, the two-fold dilution at the concentration range from 100 – 0.05 mg/mL was prepared. 100 µL of inoculum with tested strain (n=4) was added to each well, except the line H (medium purity control). Then plates were kept and sealed with parafilm. The prepared plates were incubated for five days under the same conditions as is described above (growth condition). The microplates were measured at 630 nm in the Opsys MRTM Microplate Reader before and after the incubation period. From obtained data the MIC was determined for each tested strain.

***In vitro* effect of caffeine on morphology of phototrophs and their growth by direct cells counting**

In order to monitor the potential effect of caffeine on the morphology changes and growth of phototrophs during the cultivation period (5 days), a second experiment was set up simultaneously with MIC determination in 96-well microtiter plates. This test was performed in the sterile 2 ml microtubes (set for each tested strain consisted of 8 microtubes in three repetition) for each cyanobacterium and algae strains. 250 µl of growth medium (BG11 and CM medium) was added into each microtube. Subsequently, to each microtube (12) was added a 500 µl of caffeine (diluted in growth medium) with the concentration of 100, 12.5, 1.56, 0.20 and 0.05 mg/ml. Then each set of test microtubes was inoculated with 250 µl of tested inoculum (four with cyanobacterial and one with algal species). For each tested strain the purity control of growth medium (250 µl of growth medium in microtube n. 6), purity control of caffeine (250 µl of growth medium + 500 µl of caffein contained microtube n. 7) and growth control (250 inoculum + 250 growth medium contained microtube n. 8) were prepared. 1000 µL was the final volume of each microtube. Then the microtubes were cultivated for 5 days at 23 °C and with 24h illumination (light intensity 48,6 µmol. foton m^{-2} s^{-1}). During cultivation period, the individual test tubes were opened and 10 µl of the contents were taken from each (from each microtube containing individual concentrations of caffeine) and cell numbers of the cultured strains were counted on 3rd, 4th, and 5th day by Bürker chamber and also the potential effect of caffeine on the morphology changes were studied (on 3rd, 4th, and 5th day) during the cultivation under a microscope Olympus CX23.

Statistical analyses

All experiments were performed in independent triplicate in this study. The results of MIC_{50} (MIC at which 50% of microorganisms are inhibited) and MIC_{90} (MIC at which 90% of microorganisms are inhibited) were evaluated using probit analysis ($p<0.05$) in Statgraphics Centurion XVI program (version 16.1.11). The results of percentage growth of cyanobacterial and algae strains with caffeine and its effect on morphology of tested strains was evaluated and displayed as growth curves by Microsoft Office Excel computer software.

RESULTS AND DISCUSSION

Caffeine effect on tested phototrophs

Communities of microorganisms colonizing aquatic habitats represent suitable bioindicators for pollution detection. These communities are sensitive to changes in water chemistry, as well as changes in physical conditions. Caffeine, the most common micropollutant occurring in most of the world's water ecosystems, affects the composition of aquatic communities. Exposure of communities to this very contaminant can result in a significant reduction in the biomass of microorganisms (decrease of up to 25%) and a significant change in microbial composition. An example of change in the microbial communities there may be a growth increase of the strains such as *Bacillus* spp., *Synergistia* spp., and *Actinobacteria* spp., which are resistant to caffeine. Also, some genera of fungi show resistance to caffeine (*Cladosporium* spp., *Emericella* spp., *Aspergillus* spp. and *Phoma* spp.) **(Zhang *et al*., 2016)**. Microorganisms also could degrade caffeine. Among the known species with this ability belong for example, *Aspergillus tamarii* **(**Gutiérrez-Sánchez *et al*., **2013)**, *Trichosporon asahii* **(Lakshmi and Das, 2013)**, *Pseudomonas* sp. **(Yu *et al*., 2015)** or *P. putida* **(Summers *et al*., 2011)**. **Lawrence *et al*. (2008)** point to the fact that testing one species can be very different from the results obtained from monitoring entire organisms' communities. Caffeine synergizes with many DNA-damaging substances **(Lau and Pardee, 1982)** in various organisms due to specific inhibition of ATM/ATR kinases **(Zhou *et al*., 2000).** For example, the results obtained by monitoring the toxicity of caffeine on Daphnia expressed as a mean effective concentration show a high level of 182 mg/L **(Solomon *et al*., 1996)** and for zooplankton up to 600 to 700 mg/L range **(Fent *et al*., 2006)**. However, in the study of **Brun *et al.* (2006),** they found a lower mean effective concentration of 1 mg/L against *Lemna gibba*. Moreover, caffeine has a high and stable disposal rate in water **(Li *et al*., 2020a,b)**, and therefore aquatic ecosystems may suffer from a decrease in primary production and be modified under constant contamination pressure **(Lai *et al*., 2015)**.

Based on studies of phototrophic microorganisms, in general, caffeine has an inhibitory effect on growth at specific concentrations of cyanobacteria and algae. Therefore, in this study, the potential negative effect of caffeine on the growth or morphological changes in some cyanobacterial (*Geitlerinema cf. acuminatum* (CCALA 141), *Chlorogloeopsis fritschii* (CCALA 039), *Chlorogloeopsis fritschii* (CCALA 1005), and *Synechococcus granulates* (CCALA 187)), and one algal (*Euglena gracilis* (strain Z)) strains were studied. Firstly, the MIC of caffeine was determined, and then the growth curves of tested strains depending on the tested concentration were created.

Determination of minimum inhibitory concentration (MIC)

Our results showed that caffeine strongly inhibited the growth of tested phototroph strains at relatively low concentrations. For *G. cf., acuminatum* (CCALA 141) was the lowest value of MIC_{50} estimated by probit analysis (7.26 mg/mL). The lowest MID_{90} values were found at a concentration of 26.52mg/mL also for *G. cf. acuminatum* (CCALA 141) and for *Chlorogloeopsis fritschii* (CCALA 1005) at 40.99 mg/mL after five days of cultivation. The highest value of MIC_{50} and MIC_{90} was estimated for *Euglena gracillis* (strain Z) (MIC_{50} 48.34 mg/mL and MIC_{90} 133.93 mg/mL). Algae generally tolerate high concentrations of caffeine well. They do not kill up to 200 mg/ml of caffeine. It is widely known that caffeine has antioxidant effects with the ability to induce enzymes GPR and GPX, which scavenge ROS (reactive oxygen species) at concentrations of caffeine ≥50 µg/L **(Aguirre-Martínez *et al*., 2015)**. **Čižková *et al*. (2019)** compared the cell growth of the *Chlamydomonas reinradtii* in a caffeine medium with cell growth in a nutrient medium without caffeine added. In standard conditions, the cells reproduce vegetatively *C. reinhardtii* by a series of DNA replications, each of which follows nuclear division - alternating S/M 000021 **(Coleman, 1982)**. In the study of **Čižková *et al*. (2019),** the untreated cells (caffeine-free nutrient medium) began replicating their DNA after 9 hours of the cell cycle, and after 12 hours, the cells progressed through nuclear and cell divisions. DNA replication in caffeine-treated cells (caffeine-supplemented nutrient medium) compared to untreated cells increased approximately eight times. Cell division was accelerated by about two hours in the presence of caffeine compared to untreated cells.

Table 1 Minimum inhibitory concentration (MIC_{50} and MIC_{90}) for used antibiotics able to inhibit growth of tested phototrophs (n=5) estimated by probit analysis expressed as mg/mL

Tested strains	Caffeine (mg/mL)	
	MIC_{50}	MIC_{90}
***Geitlerinema cf. acuminatum* (CCALA 141)**	7.26	26.52
p-value	*0.0000*	
***Chlorogloeopsis fritschii* (CCALA 039)**	8.76	85.06
p-value	*0.0000*	
***Chlorogleopsis fritschii* (CCALA 1005)**	10.81	40.99
p-value	*0.0000*	
***Synechococcus bigranulatus* (CCALA 187)**	9.96	52.74
p-value	*0.0000*	
***Euglena gracilis* (strain Z)**	48.34	133.93
p-value	*0.0000*	

A study by **Pollack *et al*. (2009)** reported the effects of caffeine on four species endosymbionts of coral algae, namely *Zoanthus sociatus* from the algae *Symbiodinium microadriaticum*, *Aiptasia pallida* from algae *Symbiodinium* sp., *Pseudoterogorgia bipinnata* from the algae *Symbiodinium* sp. and *Discosoma sancti-thomae* from *Symbiodinium goreaui*. In evaluating the effect of caffeine on the physiology of algae, they used two-dimensional polyacrylamide electrophoresis and peptide spectrometry to identify proteins sensitive to caffeine exposure. Heat shock proteins are among those usually affected proteins, indicating that the presence of caffeine associated with wastewater discharge into natural waters can amplify the effects of stress from other environmental factors, such as changes in ocean temperatures and pH. The results suggest that regular algal growth rates vary widely, regardless of species. Algae *S. microadriaticum* and *Symbiodinium* sp. from *Aiptasia pallida* grew the fastest. They reached the stationary phase after 15 days of growth, while *S. goreaui* algae reached the stationary phase only after 40 days and *Symbiodinium* sp. from *Pseudoterogorgia bipinnata* after 50 days of incubation. Caffeine also significantly suppressed the growth of algae during logarithmic phases. The minimum inhibitory concentration (MIC) of caffeine for algae was *S. goreaui* and S. sp. from *Pseudoterogorgia bipinnata* = 30 mg/l, *S. microadriaticum* = 50 mg/l and *Symbiodinium* sp. from *Aiptasia pallida* =

75 mg/l. Based on such results, some algae are more resistant to the effects of caffeine than others.

An example is the increasing growth of the alga *S. microadriaticum* after 22 days of incubation in the presence of caffeine, which indicates that this organism can use caffeine as a nutrient. **Nicolas** *et al***. (1980)** in their study came up with the results of nuclear repair and chloroplast DNA in the alga *Euglena gracilis* after exposure to ultraviolet radiation. They report that the repair of damaged DNA after UV irradiation is inhibited by 10 mM caffeine. They also found that caffeine at 10mM had no effect on clonal reproduction but greatly reduced the survival of these clones, while the number of green colonies remained the same. The study concludes that caffeine inhibits nuclear repair. However, DNA did not affect the repair of chloroplast DNA in any way. **Dinis** *et al***. (2021)** in their study verified the hypothesis or mechanisms of action of caffeine (CAF), and its antioxidant effects influence the toxicity of ciprofloxacin (CIP), which is used in both human and veterinary medicine. Antimicrobial substances in water ecosystems in bacteria induce the development of resistance genes **(Kümmerer, 2010)**. Conventional toxicological studies usually focus on one toxicological parameter, such as growth rate. Less often, toxic effects are evaluated in combinations of drugs **(Zhang** *et al***., 2012)**. The experiment was carried out on the microalgae *Raphidocelis subcapitata*. When observing the effect of caffeine on the growth of this species, they found that in all of them, significant inhibition was observed at caffeine concentrations from 15 to 1000 µg/L cell density. At a caffeine concentration of 1000 µg/L, they observed a decrease in cell density by 5.3 times. On the other hand, the growth rate was only visible at high concentrations of caffeine, from 100 to 1000 µg/l. In testing the CIP toxicity hypothesis, they found caffeine reduced CIP toxicity. The combination of CAF-CIP substances monitored the dynamics of microalgal growth inhibition. Cell density in the presence of CIP-CAF was significantly increased.

After evaluating the MIC, the growth curves for all strains treated with caffeine were made by recalculating the turbidity in 96-vells microtiter plates and comparing them with the control. The results were expressed as percentages of growth (Figure 1), while the red line represents the control of each tested microorganism. The growth of *G. cf. acuminatum* (CCALA 141) was completely inhibited at two tested concentrations of caffeine (100 and 50 mg/mL) (Figure 1). A slight increase of the growth was seen at a concentration of 25 mg/mL (7.20%), but at the lower tested concentration (0.05 mg/mL), the strain shoved a maximum of only 65.28 % of the growth. So according to our results, his strain was the most sensitive to caffeine tested in this study. Also, in a study by **Chapman and Meeks (1987)**, the effect of caffeine on cyanobacteria *Anacystis nidulans* was studied. The authors found that a caffeine concentration of 200 µg/mL reduced the viability of *A. nidulans* cells by about 20%. **Rempel** *et al***. (2021)** exposed *Spirulina platensis* (LEB-52) to caffeine at 1, 5, 10, 30, 50, 70 and 100 mg/L concentrations. They found that during the 20 days of cultivation, the cyanobacterium could grow in all tested concentrations of caffeine, but the production of biomass was affected. At a concentration of 100 mg/L, this strain seems resistant to caffeine. So, they concluded that caffeine does not represent a high toxic risk for the survival of aquatic microorganisms, but the highest concentrations of caffeine showed a lower level of biomass production. Moreover, some of the phototroph's microorganisms could have the potential the remediation of caffeine from wastewater.

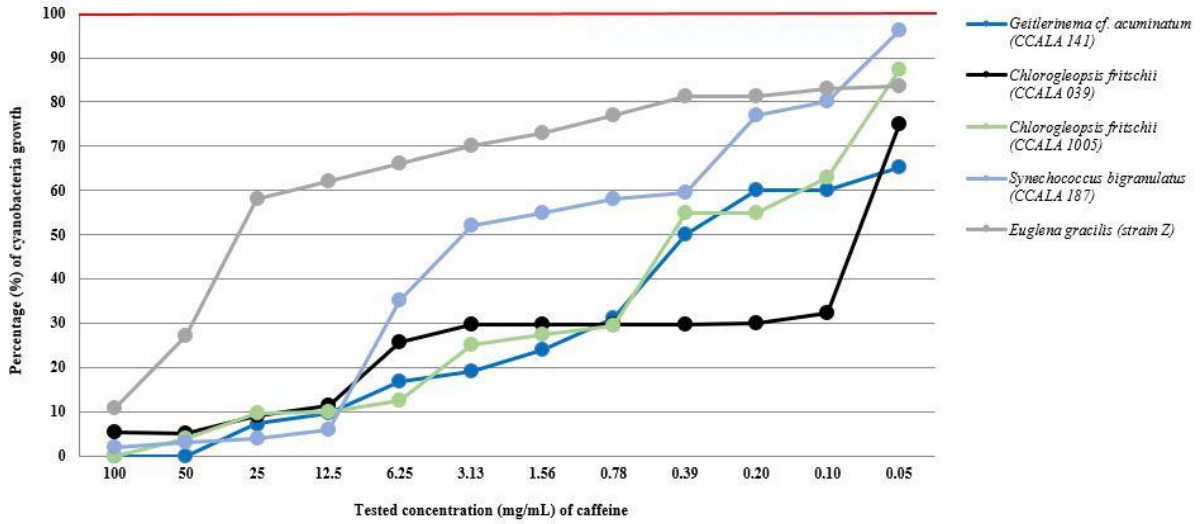

Figure 1 The growth curves mean (*n=3*) of tested phototrophs strains under treatment with caffein at different concentration (100-0.05 mg/mL) after 5 days of cultivation at 23 °C and with 24h illumination (light intensity 48,6 µmol. foton m^{-2} s^{-1}) (the red line represents the control of each of the tested microorganisms)

On the contrary, **Lawrence** *et al***. (2012)** investigated the effect of caffeine on river biofilms. To the river biofilm, which also included cyanobacteria, added concentrations of caffeine at concentrations of 10 µg/L and 5 µg/L. At a concentration of 10 µg/L, cyanobacteria in the monitored samples decrease their biomass production in the biofilm. Moreover, at a concentration of 5 µg/L, also observed a decrease in algae biomass production, while the biomass of cyanobacteria was not affected by this caffeine concentration. Also, de **Sousa** *et al***. (2021)** came to the conclusion of their study that cyanobacterial strains from a periphytic biofilm cultivated in the laboratory at a caffeine concentration of 300 µg/L were the most numerous group present among the observed organisms (Bacillariophyceae, Chlorophyceae, Cyanophyceae, Zygnemaphyceae). The complete growth inhibition of *Chlorogloeopsis fritschii* (CCALA 1005) could be seen only at a concentration of 100 mg/mL. By reducing concentrations from 50 mg/mL to 0.78 mg/mL, slow growth of the studied strain from 4 to 29.3% was seen. At the lowest caffeine concentration of 0.05 mg/mL, growth (87.40%) was observed. The growth of strain *C. fritschii* (CCALA 039) was not affected by the tested concentration, even at the highest concentration used (100 mg/mL - 5.4% of growth). At lower concentrations (0.10 - 6.25 mg/mL), the percentage growth is in the range between 25.70 - 32.30%. This strain was the most sensitive phototroph to caffeine tested in this study. In comparison with the other tested strain (*C. fritschii* (CCALA 1005), their growth was inhibited until concentration 0.10 mg/mL, where it showed a maximum of 30% growth. For example, the authors **Srivastava** *et al***. (1971)** tested the inhibitory effect of caffeine on the cyanobacterium *Anabaena doliolum*, which belongs to the same order Nostocales as *Chlorogloeopsis fritschii*. They found that its growth was not inhibited by caffeine up to a concentration of 200 µg/mL, which agrees with our results. The strain *S. bigranulatus* (CCALA 187) showed very low growth (up to 10%) at the higher tested concentration in comparison with the other tested strains. However, its growth was rapidly increased at a lower concentration (0.05 mg/mL) and had the highest measured growth value (96.30 %) from all monitored strains in this study. *Euglena gracilis* (strain Z) was the only representative of algae isolates tested in this study. This strain showed the highest tolerance to the higher concentrations of caffeine 100, 50, 25 and 12,5 mg/mL from all tested phototrophs in this study. At the highest concentration, 100 mg/ml, the growth of *E. gracilis* (strain Z) has a value of percentage growth up to 10.70%. The uniform increase of cell growth was observed until exposure to the lowest concentration (0.05 mg/mL) with a percentage growth value of 83.50%. Similar results were obtained in the study of **Sandlie** *et al***. (1980)**. The authors found that the concentration of caffeine highest than 1.55 mg/mL doesn't have a significant effect on the growth of *E. gracilis*.

Effect of caffeine on morphology and growth of tested phototrophs during the cultivation period

Microorganisms are exposed to various stresses throughout their life cycle situations in the environment **(Altermann** *et al.*, **2004)**. As an unnatural component of aquatic ecosystems, caffeine also causes stress responses in microorganisms that depend on its concentration. Organisms subsequently develop against these unfavourable and unnatural phenomena adaptive mechanisms that subsequently help them grow and survive in the environment (**Jydegaard-Axelsen** *et al***., 2005**). Belong adaptive mechanisms for stressful situations include, for example, regulation of gene expression, which can subsequently lead to modifying physiological and phenotypic expression characteristics. Based on studies, it is assumed that changes in morphology are one from adaptation mechanisms that prolong the bacteria's survival in unfavourable or stress-inducing conditions (**Piuri** *et al***., 2005; McMahon** *et al***., 2007**). Thus, the next step in this study was to monitor the potential morphological changes of the tested phototrophs after five days of cultivation with different concentrations of caffeine. The effect of caffeine on selected microorganisms was observed by direct counting in the

Bürker chamber at five selected concentrations (0.05, 0.20, 1.56, 12.5 and 100 mg/mL) of caffeine.

The growth of *G. cf. acuminatum* (CCALA 141) was significantly stimulated on the 3rd day of cultivation compared with the control at the lowest concentration tested (0.05 mg/mL). The rest of the concentrations inhibited the growth of this strain. For comparison, in the study of **Lambert** *et al.* **(1980)**, the authors observed the influence of caffeine on the growth of *Gloeocapsa alpicola* when the cultures are exposed to UV radiation. The experiment results indicate that caffeine is generally toxic to *G. alpicola*. But they found that the caffeine concentration under 0.15 mg/mL did not affect the loss of photo reversibility of *G. alpicola*, which was irradiated with UV light. A caffeine concentration of 2 mg/mL was toxic to cells of *G. alpicola* not exposed to UV light. This concentration of caffeine inhibited the loss of photo reversibility, caused damage under UV radiation, and reduced their viability, which is thought to impede pre-replication DNA repair. In conclusion, they state that cells were more sensitive to UV killing in the presence of caffeine.

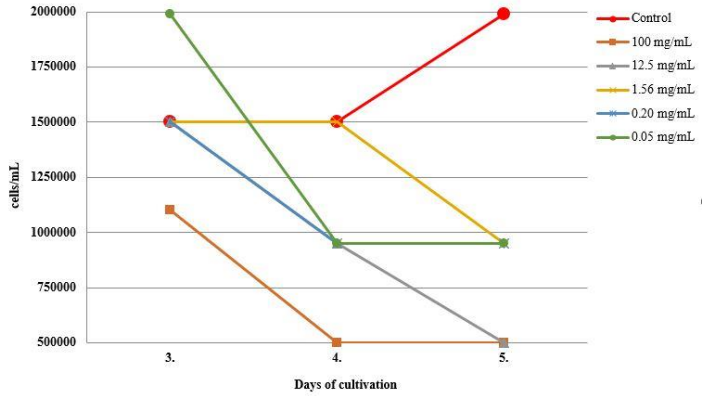

Figure 2 The growth curve of *Geitlerinema cf. acuminatum* (CCALA 141) at selected concentrations (0.05, 0.20, 1.56, 12.5 and 100 mg/mL) of caffeine during the exponential phase of growth on 3rd, 4th and 5th days of cultivation

When the morphological changes in *G. cf. acuminatum* (CCALA 141) was monitored, the changes in the sizes of cells of the microorganism were visible at concentrations from 1.56 – 12.5 mg/mL (Figure 3A and 3B). The short filaments with longer cells with dark green colour were visible. The longer filaments were also visible, but these were only dead parts or parts already subject to decomposition. No live cells were visible at the highest tested caffeine concentration (100 mg/mL). At lower concentrations (1.56 mg/mL) (Figure 3C), the long filaments of deep green colour were visible under the microscope.

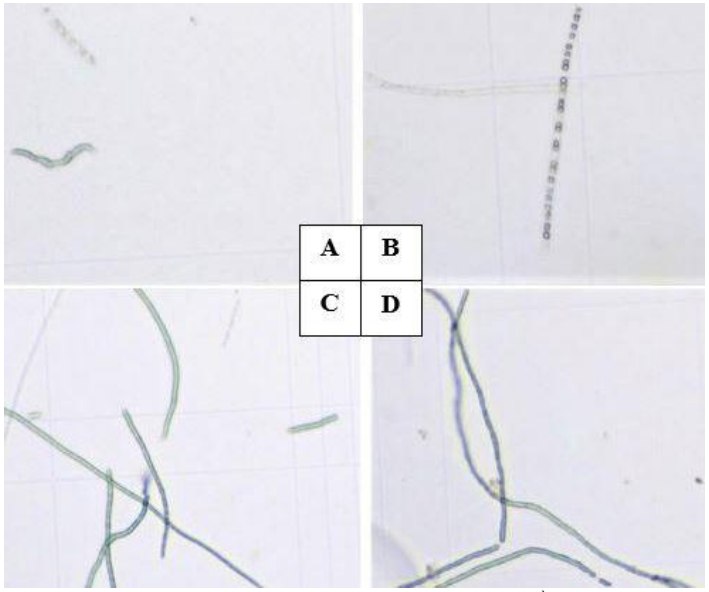

Figure 3 *Geitlerinema cf. acuminatum* (CCALA 141) on the 3rd day of cultivation: A – with the addition of caffeine 12.5 mg/mL; B – with the addition of caffeine in concentration 1.56 mg/mL; C – with the addition of caffeine at a concentration of 0.20 mg/mL; D – control, without the addition of caffeine

In case of *C. fritschii* (CCALA 1005) The two lowest caffeine concentrations (0.05 and 0.20 mg/mL) significantly stimulated its growth on 3rd day of cultivation in comparison with the control similar to the previous specie (Figure 4). The other tested concentrations had an inhibitory effect on this strain. Also, the growth of *C. fritschii* (CCALA 039) was stimulated on 3rd day of cultivation. However, its growth was inhibited more slowly by other tested concentrations of caffeine compared to *C. fritschii* (CCALA 1005) (Figure 5).

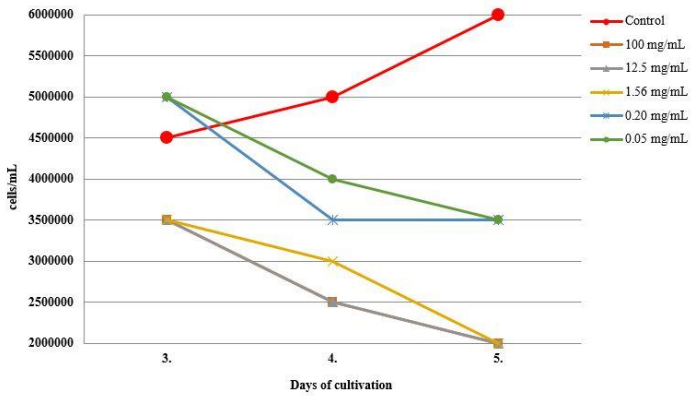

Figure 4 The growth curve of *Chlorogloeopsis fritschii* (CCALA 1005) at selected concentrations (0.05, 0.20, 1.56, 12.5 and 100 mg/mL) of caffeine during the exponential phase of growth on 3rd, 4th and 5th days of cultivation

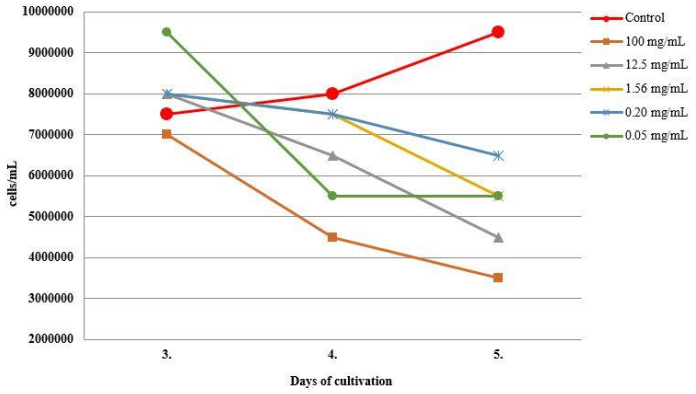

Figure 5 The growth curve of *Chlorogloeopsis fritschii* (CCALA 039) at selected concentrations (0.05, 0.20, 1.56, 12.5 and 100 mg/mL) of caffeine during the exponential phase of growth on 3rd, 4th and 5th days of cultivation

In control sample the cells of *Chlorogloeopsis fritschii* (CCALA 1005), were still dividing, they had a round shape cells, created small compact colonies with four to eight cells and had a deep green color even on the 5th day (Figure 6A and C). In the presence of caffeine (12.5 mg/ml) they still formed colonies but, in this case, they already lost color (Figure 6B and D). On the 3rd (Fig. 6A, B) day of cultivation the cells clustered into the typical multiple packages like colonies that are characteristic of *Chlorogloeopsis* sp. On the 5th day of cultivation with caffeine the cell colonies are not formed, but the cells were separate. Similar morphological changes were observed in *Chlorogloeopsis fritschii* (CCALA 039) (Figure 7A and B). In the control sample cultured without caffeine, the cells formed many colonies with a deep green colour but in presence of caffeine (12.5 mg/mL) occurred a chain-like colony with a significantly weaker, green colour. Colonies also contains death cells and was visible an absence of mucilage sheaths around cells. For comparison *C. fritschii* (CCALA 039) on the 5th day of cultivation produced more cells than *C. fritschii* (CCALA 1005). This observation also coincides with the growth curves of these microorganisms (Figure 4 and 5).

So, the presence of caffeine in water is undesirable and can have a very negative effect on the growth of cyanobacteria. For example, authors **Levine and Thiel (1987)** quantitated DNA repair in several strains of *Anabaena* spp. by measuring the reactivation of UV-damaged cyanophage. They found that cells of tested *Anabaena* spp. strains were resistant to several hundred joules of UV irradiation per square meter. But many other authors found that in several cyanobacterial strains, the sensitivity to UV – irradiation increased in the presence of caffeine **(Srivastava *et al*., 1971; Asato, 1972; Williams *et al*., 1979).**

The growth of *Synechococcus bigranulatus* (CCALA 187) was stimulated on the 3rd day of cultivation at concentrations of 0.05 mg/mL compared with the control sample (Figure 8). Concentrations 12.5 and 100 mg/mL had an inhibitory effect on this strain from the beginning. The growth of control (untreated with caffeine) showed a sharp increase in biomass production on the 5th day of cultivation.

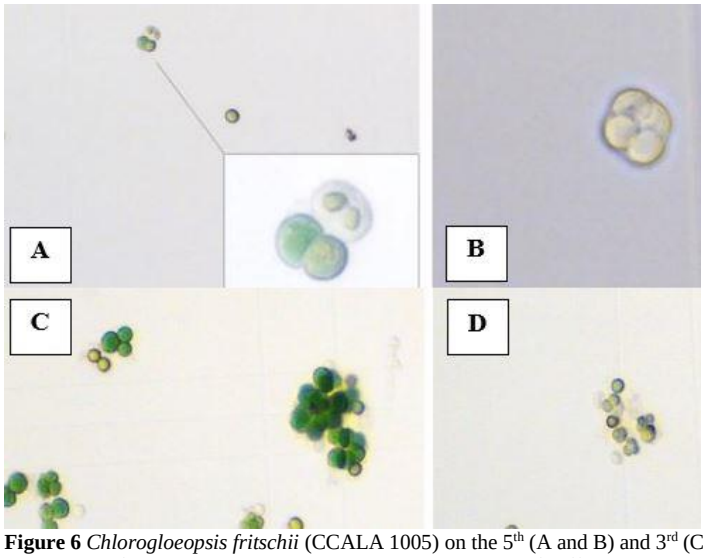

Figure 6 *Chlorogloeopsis fritschii* (CCALA 1005) on the 5th (A and B) and 3rd (C and D) day of cultivation: A – control, without the addition of caffeine; B – with the addition of caffeine in concentration 12.5 mg/mL; C – control, without the addition of caffeine; D – with the addition of caffeine at a concentration of 12.5 mg/mL

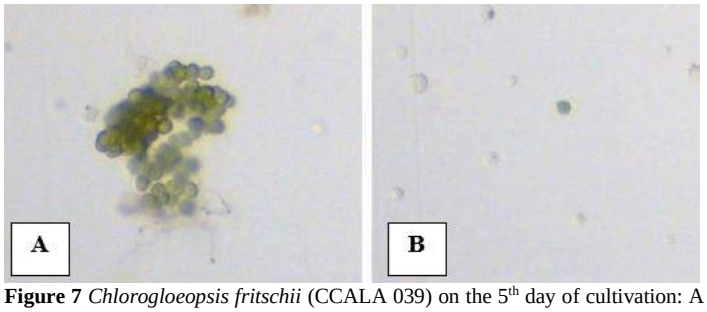

Figure 7 *Chlorogloeopsis fritschii* (CCALA 039) on the 5th day of cultivation: A – control, without the addition of caffeine; B – with the addition of caffeine in concentration 12.5 mg/mL

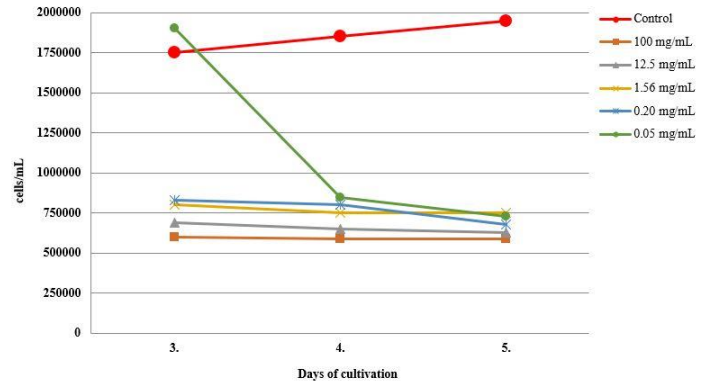

Figure 8 The growth curve of *Synechococcus bigranulatus* (CCALA 187) at selected concentrations (0.05, 0.20, 1.56, 12.5 and 100 mg/mL) of caffeine during the exponential phase of growth on 3rd, 4th and 5th days of cultivation

Caffeine caused a significant difference in the cell size of *Synechococcus bigranulatus* (CCALA 187). Small cells were presented at the highest concentration of caffeine 100 mg/mL (Figure 9A), and in the control sample (Figure 9D). At concentrations of 12.5 mg/mL (Figure 9B), some cells were elongated up to 10-fold. At a lower concentration of 0.20 mg/mL (Figure 9C), the cells were longer again than the control but only by two times its length.

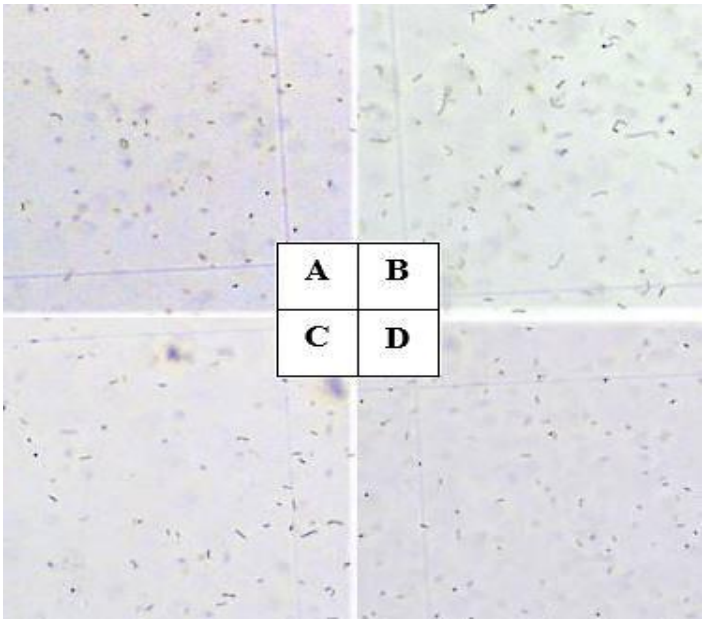

Figure 9 *Synechococcus bigranulatus* (CCALA 187) on the 3rd day of cultivation: A – with the addition of caffeine in concentration 100 mg/mL, B - with the addition of caffeine in concentration 12.5 mg/mL, C – with the addition of caffeine in concentration 0.20 mg/mL; D – control, without the addition of caffeine

The strain *E. gracilis* (strain Z), as the only representative of algae in this experiment, showed considerable stimulation of growth on the initial days of cultivation (3rd) at the two lowest caffeine concentrations of 0.05 and 0.20 mg/mL (Figure 10). Higher concentrations of caffeine from 1.56 mg/mL had an inhibitory effect on the microbial growth of this strain compared with the control sample. Growth stimulation at lower concentrations of caffeine was observed in all species tested in this study. Our results agree with **Čížková** *et al***. (2019)**. In their study, *Chlamydomonas reinhardtii* cells treated with caffeine divided about two hours faster. The amount of their DNA was increased up to eight times until there was no depletion of nutrients, and the dying phase did not occur. It could happen because caffeine causing that the completion of DNA replication and mitosis to be uncoupled **(Schlegel** *et al***., 1986; Amino and Nagata, 1996)**. Caffeine can accelerate mitosis **(Kumagai** *et al***., 1998a; Kumagai** *et al***., 1998b; Moser** *et al***., 2000)** and overrides replication and potential damage checkpoints DNA in different systems **(Pelayo** *et al***., 2001; Weingartner** *et al***. 2003)** due to of specific inhibition of ATM/ATR kinase **(Zhou** *et al***., 2000; Moser** *et al***., 2000)**. In the case of a study by **Čížková** *et al***. (2019)**, the effect of caffeine on the species *Chlamydomonas reinhardtii* was only partial, and its presence did not induce faster nuclear fission. It can be caused either by using a low concentration of caffeine or by the presence of a possible alternative DNA checkpoint pathway that is either insensitive to caffeine or requires its higher concentrations.

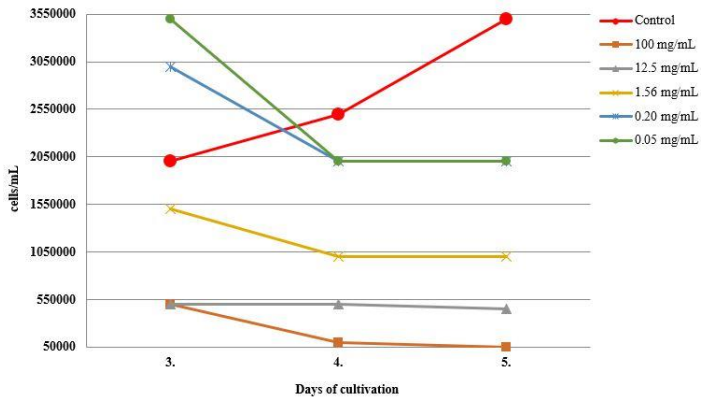

Figure 10 The growth curve of *Euglena gracilis* (strain Z) at selected concentrations (0.05, 0.20, 1.56, 12.5 and 100 mg/mL) of caffeine during the exponential phase of growth on 3rd, 4th and 5th days of cultivation

The cells of *Euglena gracilis* (strain Z) (Figure 11) untreated by caffeine had an elongated shape with visible flagella and stigma. In presence of caffeine from 1.56 mg/L to 100 mg/mL were non-motile, or very little motile, most of them had lost their chlorophyll and their shape was spherical. At a caffeine concentration of 1.56 mg/mL, cells also became visible with an elongated shape. In the other days of cultivation, the morphology of the algal cells did not change, only number of cells was changed. The visible dead cells were observed at concentrations from 12.5 mg/mL.

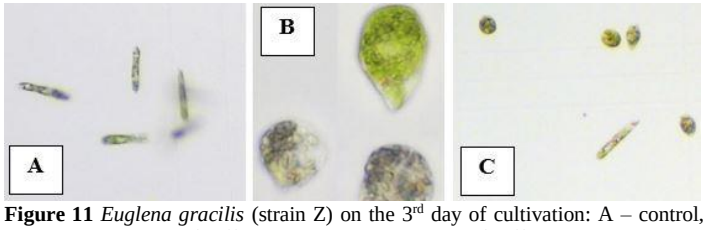

Figure 11 *Euglena gracilis* (strain Z) on the 3rd day of cultivation: A – control, without the addition of caffeine, B – with the addition of caffeine in concentration 100 mg/mL, C – with the addition of caffeine in concentration 1.56 mg/mL

CONCLUSION

In the present study, the inhibitory effect of caffeine on selected phototrophic microorganisms was evaluated. Our results showed that caffeine, at relatively low concentrations, strongly inhibited the growth of the phototrophic strains tested. The most sensitive strains were *Geitlerinema cf. acuminatum* (CCALA 141) and *Chlorogloeopsis fritschii* (CCALA 1005). *Euglena gracilis* (strain Z) shows higher resistance to caffeine. A growth-stimulating effect was also observed in the strains studied at lower caffeine concentrations (0.05 and 0.20 mg/mL). This study evaluated the potential impact of different caffeine concentrations on the morphological changes of tested strains. In the cyanobacterial strains, changes in cell size were observed. In all strains, the cells were mainly enlarged. In *Chloreogleopsis fritschii* (for both tested strains), there was a change in its typical colonies. In the species, *Euglena gracilis* (strain Z), changes in shape and slowing of movement were observed. At high caffeine concentrations, the elongated, spindle-shaped cells became oval. In general, the studied stains are sensitive to higher concentrations of caffeine. However, lower concentrations in studied strains stimulate their growth (this was reported in part studied their morphology). The reported concentrations in nature, then, could support their presence in microbial biofilms, and this ability can give them an advantage in different types of aquatic habitats. At the same time, it can be argued that the species studied are potentially suitable for experiments aimed at degrading caffeine in nature and studying their use as remediation agents.

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